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Type I collagen prevents amyloid aggregation of hen egg white lysozyme

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ABSTRACT

Both collagen and amyloidogenic proteins have an inherent ability to undergo a self-assembly process leading to formation of supramolecular structures. Though our understanding of collagen–amyloid link is very poor, a few experimental evidences have indicated the protective nature of collagen against amyloid fibril formation. To further our understanding of collagen–amyloid relationship, we have explored the role of type I collagen on amyloid-aggregation of lysozyme. Thioflavin-T assay data indicated strong inhibition of both spontaneous and seeded aggregation of lysozyme by collagen. Both chemical and thermal denaturation experiments have showed increased lysozyme stability in the presence of collagen. However, the presence of collagen did not alter lysozyme activity. These findings confirm that type I collagen is capable of blocking or interfering with the amyloid aggregation of lysozyme, and the results may have significant implications for the design of collagen based therapeutics against aggregation of disease linked amyloidogenic proteins.

1. Introduction

Self-association of protein molecules into higher order assemblies such as amyloid fibrils and collagen assemblies is a fundamental process in biology. Nature has employed the self-assembly process of triple-helical collagen molecules to generate higher order supramolecular assemblies which are vital to both structural and functional properties of the extra cellular matrix (ECM) [1]. However the process of aggregation of many proteins into amyloid fibrils is mostly linked to a number of pathological complications including a series of neurodegenerative diseases. Until now around 20 different proteins including huntingtin, α-synuclein, amylin, β2-microglobulin, and lysozyme have been found to form β-amyloid aggregates leading to severe pathologies [2,3]. On the other side however, amyloid fibril formation in some cases is beneficial and essential for certain biological functions such as protein storage granules used by mammals [4].

Since most amyloids are directly or indirectly linked to diseases, there is an increasing interest in finding a straightforward strategy to prevent such amyloid aggregation of proteins. Though there is no direct report on collagen–amyloid relationship, some studies have revealed the protective roles of collagen and collagen model peptides against amyloid aggregation of proteins [5–8]. In an *in vitro* investigation Kiuchi et al. [6] have shown that type IV collagen acts as a potent inhibitor against amyloid aggregation of Aβ-protein. A recent report has revealed that type VI collagen is capable of preventing Aβ neurotoxicity [7]. The same study has also shown that a possible interaction between soluble type VI collagen molecules and Aβ oligomers is behind the prevention of neurotoxicity [7]. A collagen model peptide $(Pro\text{-}Hyp\text{-}Gly)_{10}$ is found to suppress the aggregation process of an amyloidogenic peptide GNNQQNY [5]. Molecular interactions between collagenous Alzheimer amyloid plaque component (CLAC) and Aβ fragments have been reported to reduce Aβ fibril elongation [8]. Investigations have also revealed that the basement membrane components including collagen can trigger disassembly of Aβ amyloid fibrils [9]. Since all these observations have apparently indicated the protective role of collagens against amyloid aggregation of proteins, a mechanistic understanding of amyloid–collagen interrelationship is very important for both fundamental and applied research.

Among different types of collagen, type I is one of the most abundant forms and it is found in tissues as regular D-periodic fibrils [10,11]. In addition to fibrillar collagens many non-fibrillar forms of collagens are present which make up vital tissue components in the body system. Such non-fibrillar collagens include networks of type IV collagen in the basement membrane [12–14], hexagonal networks of type VIII collagen in the subendothelial layers [15], antiparallel arrays of type VII collagen [16] and microfibrils of type VI collagen in all connective tissues [17].

Protein–protein interactions are critical to many mechanisms in biology and the relationship between triple-helical collagen proteins and globular proteins has not been widely studied yet. Here we have explored the effect of type I collagen, the most

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abundant form of collagen, on the amyloid aggregation of hen egg white (HEW) lysozyme. HEW lysozyme is one of the most commonly studied proteins for understanding the molecular structure, folding, and functional properties of globular proteins [18]. Lysozyme is known to undergo a process of amyloid aggregation under a condition where its partially folded intermediates are highly populated [19–21]. Such conditions include acidic pH, presence of denaturants, and incubation of lysozyme at a temperature below its transition temperature [19–22]. Hence, hen egg white lysozyme becomes one of the widely studied model proteins for understanding the amyloid fibril formation of globular proteins.

To further our understanding of collagen amyloid relationships, we have investigated the effect of type I collagen on the amyloid aggregation of hen egg white lysozyme. Here, we report the inhibition effect of type I collagen on the acid induced amyloid aggregation of hen egg white lysozyme. In addition, we have investigated the effect of collagen on the biological activity and conformational stability of lysozyme using different spectroscopic techniques.

2. Materials and methods

2.1. Reagents

Lyophilized cells of *Micrococcus lysodeikticus*, Thioflavin-T (ThT), glycine and NaCl were obtained from Sigma–Aldrich. Hen egg white lysozyme (lyophilized powder), 10× phosphate buffer saline (PBS) solution, guanidine hydrochloride (GnHCl) are procured from HIMEDIA (India). Type I collagen (Rat Tail Tendon) was a gift from Dr. Madhan Balaraman's laboratory at Central Leather Research Institute, India, [23]. All other analytical reagent grade chemicals were purchased either from HIMEDIA or from Sigma–Aldrich. PBS buffer was used for pH 7.4 and 10 mM glycine–HCl buffer was used for pH 2.0. Measurements of lysozyme concentration and activity were carried by using Varian Cary-4000 UV–vis spectrophotometer. Lysozyme concentration was determined spectrophotometrically using extinction coefficient at $\lambda = 280$ nm of 2.63 lg^{-1} cm^{-1}.

2.2. Fluorescence measurements

A Perkin Elmer LS 55 fluorescence spectrometer was used for intrinsic tryptophan fluorescence measurements of proteins in the presence and absence of collagen. All measurements were carried out at 25 °C with an excitation wavelength of 295 nm. All the spectra were baseline subtracted. Thioflavin-T binding assays were carried out by using established protocol [24] where the Thioflavin-T sample was excited at 440 nm with a slit width of 2.5 nm and the emission was observed at 490 nm with slit width of 5 nm.

2.3. Circular dichroism spectroscopy

For the CD experiments, JASCO CD spectrometer (model J-815-150 L) with attached Peltier temperature controller was used. The protein concentration and path length of the cell used were 0.25 mg ml^{-1} and 2 mm, respectively. Each plot represented here is an average of three accumulated plots. Thermal unfolding experiments was performed by recording the spectra from ~15 °C to ~90 °C with scanning rate of 2 °C min^{-1}.

2.4. Assay of lysozyme activity

The activity of lysozyme was determined against *M. lysodeikticus* using the turbidometric method [25]. The decrease in turbidity of a 1-ml bacterial-cell suspension (0.3 mg ml^{-1}) in different buffers (50 mM phosphate buffer at pH 6.5 and 10 mM glycine–HCl buffer at pH 2.0) was monitored after the addition of 0.1 ml of an appropriately diluted lysozyme solution. To the reference cell, 0.1 ml of lysozyme solution was added. The decrease in absorbance was monitored every 1 s during a total incubation of 3 min at 450 nm using spectrophotometer. Activity of the enzyme was measured in the presence of collagen and all experiments were repeated at least three times.

2.5. Amyloid aggregation of lysozyme

Amyloid aggregation was achieved by incubation of lysozyme (10 mg ml^{-1}) in 10 mM glycine–HCl buffer at pH 2.0 containing 130 mM NaCl [24,25]. The protein solution was incubated at 58 °C and small aliquots are taken out at appropriate intervals to conduct the Thioflavin-T binding assay as described in the text. We also determined the concentration of soluble protein at selected time points. For conducting seeded amyloid aggregation reactions, 5% (weight/weight) of the preformed amyloid fibrils of lysozyme was used as seed.

3. Results

3.1. Amyloid aggregation of hen egg white lysozyme

We studied aggregation of lysozyme at pH 2.0 at 58 °C by following the established protocol [24,25]. Kinetics of amyloid

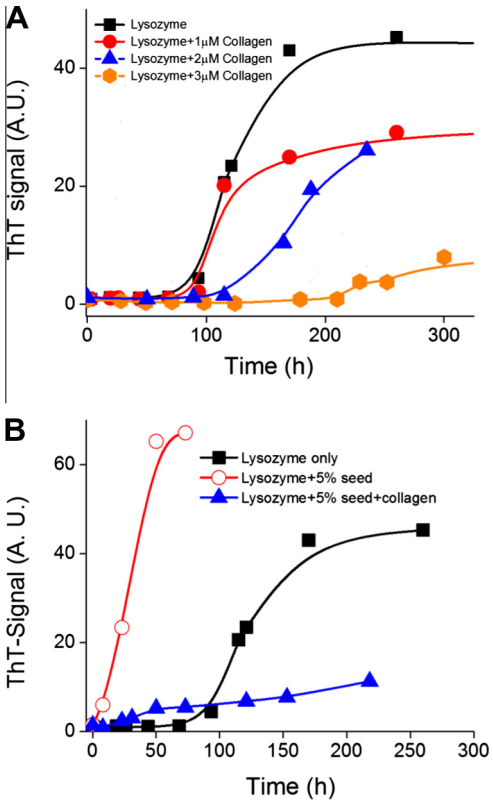

Fig. 1. Effect of collagen on lysozyme amyloid aggregation. (A) Inhibition of hen egg white lysozyme amyloid aggregation by type I collagen. Lysozyme solution (pH 2.0 and 10 mg ml^{-1}) is incubated at 58 °C and the amyloid aggregation was observed by following the Thioflavin-T signal of the sample at different time points (see methods): Only lysozyme (■); lysozyme +1 μM collagen (●), lysozyme +2 μM collagen (◀) and lysozyme +3 μM collagen (⬢). (B) Inhibition of seeded amyloid aggregation of lysozyme in presence of collagen. Only lysozyme (■), lysozyme + seed (○), lysozyme + seed + 3 μM collagen (▲). Seed is the preformed lysozyme fibrils and amount of seed used in the experiments was maintained at 5% (weight/weight). All the data showed here are the average values of at least three independent measurements.

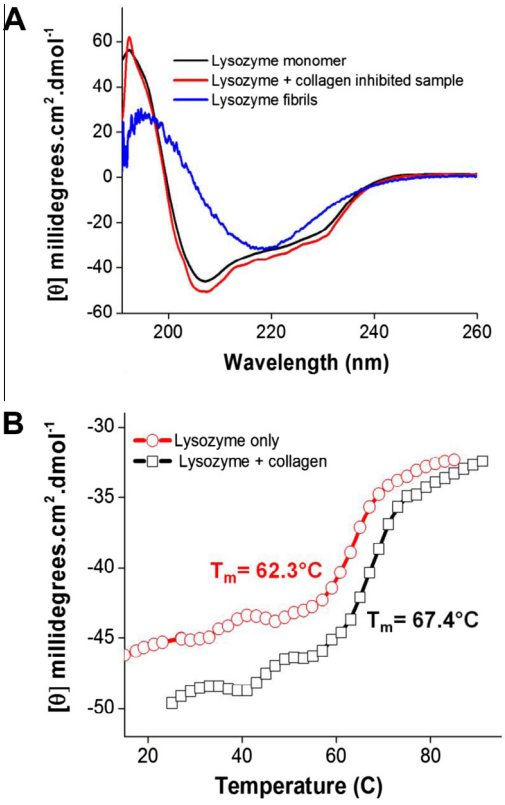

Fig. 2. Effect of collagen on lysozyme conformation and stability by circular dichroism (CD). (A) CD spectra of lysozyme samples: lysozyme monomers before aggregation (—), lysozyme amyloid fibrils aggregation (—), inhibited lysozyme sample in the presence of collagen (—). (B) Thermal unfolding of hen egg white lysozyme in the absence (○) and the presence (□) of type I collagen. Temperature scan rate for the thermal unfolding experiments was maintained at 2 °C/min. The value of transition temperature (T_m) of lysozyme was 62.3 °C and in the presence of collagen the T_m was increased to 67.4 °C.

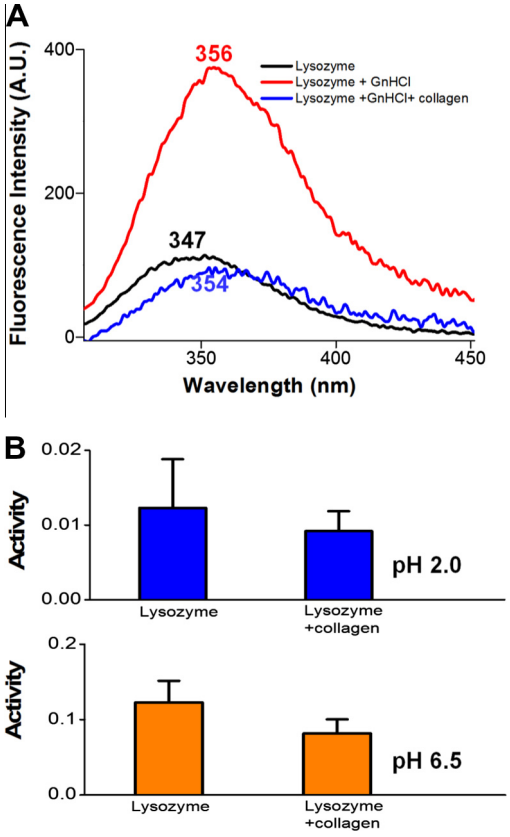

Fig. 3. (A) Intrinsic tryptophan fluorescence spectra of lysozyme: lysozyme only (—), lysozyme + 5 M GnHCl (—), lysozyme + collagen + 5 M GnHCl (—). (B) Biological activity of lysozyme in the presence of collagen. Catalytic activity of lysozyme both in the presence and absence of collagen was performed at pH 2.0 (upper panel) and at pH 6.5 (lower panel). All measurements were obtained at 25 °C and the ratio of lysozyme to collagen was maintained at 100:1. Each plot shown from both fluorescence and activity measurements represents the average value of two to three independent measurements.

formation was obtained by monitoring the Thioflavin-T (ThT) reading of the sample at regular intervals (Fig. 1A). Lysozyme in the absence of collagen showed a rise in the Thioflavin-T signal with a lag phase of ~100 h confirming the formation of amyloid fibrils (■). However, significant inhibition of aggregation was observed in the presence of collagen. The inhibition effect was studied at three different concentrations of collagen which are 1000:1, 350:1, and 250:1 M ratio of lysozyme to collagen (Fig. 1A, ●▲⬢). Lag time of the lysozyme amyloid formation increased with the increasing concentration of collagen in the sample (Fig. 1A). When collagen was present at 250:1 M ratio of lysozyme to collagen aggregation lag time was delayed to ~210 h after which a slight aggregation was observed. Even after ~700 h of incubation only ~10% of the total soluble lysozyme underwent aggregation. This result was confirmed by the measurement of concentration of soluble lysozyme monomers in the sample (data not shown).

3.2. Inhibition of seeded elongation of lysozyme amyloid aggregation

Since type I collagen showed a strong inhibition effect on lysozyme amyloid aggregation, as a next step we tried to explore its effect on a seeded aggregation reaction of lysozyme. When ~5% (weight/weight) preformed lysozyme amyloid fibrils (or seeds) are added to monomers of lysozyme solution, a rapid aggregation was observed without any lag phase (Fig. 1B, ○). However, in the presence of collagen (at 250:1 M ratio of lysozyme to collagen) a strong inhibition effect was observed where the lag time was delayed to ~30 h after which a slight rise in the Thioflavin-T signal was observed (Fig. 1B, ▲). Again we confirmed the aggregation inhibition by determination of the concentration of the soluble protein at regular intervals (Data not shown). Suppression of seeded aggregation reaction supports the inhibition ability of type I collagen against lysozyme amyloid aggregation. It is likely that the unfolded collagen chains are either interacting with lysozyme monomers and lysozyme fibrils (seeds) or to both these species preventing the onset of the aggregation process.

3.3. Effect of collagen on lysozyme activity

Since strong inhibition of lysozyme amyloid aggregation was observed in the presence of collagen, we then tried to explore whether collagen molecules have any effect on the biological activity of lysozyme. Lysozyme activity assays were carried out following established protocol in previous work [25]. At pH 6.5, catalytic efficiency of lysozyme is believed to be optimal, and we noticed no change in the presence of collagen (Fig. 3B, lower panel). Next we tried to see the effect of collagen on lysozyme activity at low pH. At pH 2.0 we observed ~10% of the optimal activity of lysozyme (observed at pH 6.5) and addition of collagen to the sample did not alter the catalytic activity of lysozyme (Fig. 3B, upper panel). Observed results suggest that the interaction between collagen chain and lysozyme does not promote regaining of the active site cleft.

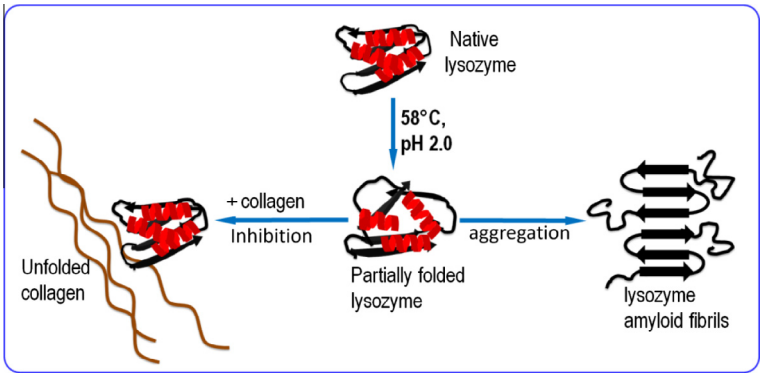

Fig. 4. Schematic representation of the inhibition of lysozyme amyloid aggregation in the presence of collagen.

3.4. Effect of collagen on conformational stability of lysozyme

To understand the effect of collagen on the conformational properties of lysozyme both CD and intrinsic tryptophan fluorescence measurements were performed. CD spectra of lysozyme monomers before aggregation showed a typical signature seen for lysozyme's native structure (Fig. 2A, black line) and after completion of the aggregation process the CD spectra indicated the presence of only β-structured species (Fig. 2A, blue[1] line). This result confirmed the conversion of soluble lysozyme monomers into cross-beta amyloid fibrils. Next, we carried out the CD measurement of the lysozyme sample from an inhibited reaction (lysozyme/collage ratio = 250). In an inhibited reaction (in the presence of collagen) the CD spectra of lysozyme (Fig. 2A, red line) looked almost identical to the native lysozyme CD spectra (Fig. 2A, black line). This indicated that the presence of collagen can promote the retention of the native structure of lysozyme.

To further understand whether collagen has any effect on thermal stability of lysozyme we conducted thermal denaturation experiments of lysozyme in the presence of collagen. Thermal melting of lysozyme was recorded by monitoring the CD signal at 207 nm. In the presence of collagen the transition temperature (T_m) of lysozyme was increased from 62.3 °C to 67.4 °C. Such an increase of T_m values suggests the gain of conformational stability of lysozyme in the presence of collagen.

To check the refolding capacity of type I collagen on a chemically denatured lysozyme molecule, experiments were performed with 5 M GnHCl treated lysozyme. Chemically denatured lysozyme showed a red shift of ~9 nm as the emission maxima (λ_{max}) changed from 347 nm to 356.5 nm with enhanced fluorescence intensity (Fig. 3A). However, in the presence of collagen (lysozyme/collagen molar ratio = 250) we observed a substantial decrease in the intensity accompanied by a slight blue shift of ~2 nm) of λ_{max} (Fig. 3A, blue[2] line). This result is good evidence that collagen has the ability to alter the conformation of the lysozyme while inhibiting the protein's aggregation. Both CD and fluorescence data indicate that in the presence of collagen lysozyme is gaining conformational stability.

4. Discussion

The current study clearly indicates that type I collagen is capable of preventing amyloid aggregation of lysozyme at pH 2.0 and at 58 °C. It is believed that increase in the population of the partially folded species of lysozyme is a major factor for the onset of its amyloid aggregation [24]. Many results have indicated that β-domain of the protein gets preferentially unfolded which later enhances intermolecular interactions leading to formation of β-amyloid fibrils [29–32]. Intermolecular association between such partially folded species is believed to be the cause for the amyloid aggregation. It has been reported that when the populations of the intermediate states become very high (>90%), the formation of amyloid fibrils is strongly favored [24,25]. As the process of fibril formation requires a critical concentration of amyloid-prone intermediates, even a small change in the population of such species can effectively trigger or suppress the amyloid aggregation of proteins [36].

One of the reasons behind the inhibition effect of collagen could be due to the interaction between the collagen and the partially folded species of lysozyme. There is a possibility that collagen–lysozyme interaction would stabilize the partially folded intermediate species and alter their conformation into a more native like structure. This assumption is supported by the CD data where we observed a ~5 °C increase of the transition temperature of lysozyme in the presence of collagen (Fig. 2B). Further, fluorescence data (Fig. 3A) also showed that a chemically denatured lysozyme could regain its lost conformational stability in the presence of collagen.

The molecular structure of collagen has a characteristic triple helical conformation, consisting of three polyproline II like chains supercoiled around a common axis by interchain hydrogen bond [26–28]. The type I collagen (from Rat Tail Tendon) molecule which is used in the current study consists of three chains, two α1 (1453 residues each) chains and one α2 chain (1372 residues). Since the transition temperature of type I collagen is about 42 °C [33], all the triple-helical molecules at 58 °C are expected to exist as unfolded individual chains. Therefore in this study the collagen–lysozyme interaction leading to inhibition effect is between the unfolded chains of the collagen and the partially folded lysozyme species as shown in the schematic illustration in Fig. 4. It is also possible that the exposed residues of partially folded lysozyme and the unfolded chains of collagen may interact with each other. One of the key factors for amyloid aggregation is that the condition under which aggregation happens should favor the intermolecular interactions including hydrogen bonding [37]. Interaction between an unfolded collagen chain and a partially folded lysozyme could be possible through their exposed hydrophobic and aromatic residues. One of such critical interactions may include aromatic–proline interaction [34,35] since collagen chains have a high content of the imino acids (proline and hydroxyproline). Analysis of the amino acid sequence of the collagen chains (Supplementary Fig. S1) indicates the presence of large number of proline residues and a substantial number of aromatic and hydrophobic residues. Thus, aromatic–proline interactions as well as hydrophobic interactions

[1] For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

[2] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

between collagen chain and the amylodogenic segment of lysozyme might be critical to the inhibition mechanism. To gain more insight into the collagen–lysozyme interaction, we have investigated the effect of lysozyme on collagen fibril formation at 37 °C in PBS. Results indicate no effect of lysozyme on the onset of collagen fibril formation (Supplementary Fig. S2). Since at 37 °C lysozyme would retain its native conformation and collagen molecules would exist as intact triple helices, a lysozyme–collagen interaction seems to be less favorable. Thus, we believe that the intermolecular interaction between unfolded chains of collagen and partially folded lysozyme species is more favorable and it could be one of the reasons behind the inhibition effect. Moreover, our data suggest that type I collagen is protective against amyloid aggregation of lysozyme and such results may have implications for collagen based therapeutics against neurodegenerative diseases associated with amyloids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.135.

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